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(71) Applicant *(for all designated States except US)*: **NOVOZYMES A/S** [DK/DK]; Krogshøjvej 36, DK-2880 Bagsværd (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CHRISTENSEN, Morten, Würtz** [DK/US]; 2912 Coxindale Dr., Raleigh, NC 27615 (US). **HOLM, Hans, Christian** [DK/DK]; Hellerupgaardvej 7, DK-2900 Hellerup (DK). **ABE, Kyoko** [JP/JP]; 3-5-17, Mihama Urayasu-shi, Chiba-ken, Chiba 279-0011 (JP).

(74) Common Representative: **NOVOZYMES A/S**; Patents, Krogshøjvej 36, DK-2880 Bagsværd (DK).

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(54) Title: FAT SPLITTING PROCESS

(57) Abstract: A process for hydrolyzing a triglyceride into glycerol and fatty acid comprises contacting the triglyceride and water consecutively or simultaneously with a positionally specific microbial lipase and a positionally non-specific lipase. The combination of lipases has a synergistic effect on the hydrolysis of triglycerides, so that a high degree of hydrolysis of the triglycerides can be obtained.

FAT SPLITTING PROCESS

FIELD OF THE INVENTION

The present invention relates to enzymatic fat hydrolysis (fat splitting), i.e. the hydrolysis of triglycerides (fat or oil) into glycerol and fatty acid.

BACKGROUND OF THE INVENTION

It is known to hydrolyze triglycerides by use of a lipase. JP 1-291798 A, JP 2-13389 A and 3898130 disclose the use of two lipases for hydrolysis of oil. US 5061498 discloses treatment of fats and oils with two lipases in the presence of a small amount of water to convert mono- and diglycerides included in the fats and oils into triglycerides.

SUMMARY OF THE INVENTION

The inventors have found that a certain combination of lipases has a synergistic effect on the hydrolysis of triglycerides. Accordingly, the invention provides a process for hydrolyzing a triglyceride into glycerol and fatty acid, comprising contacting the triglyceride and water consecutively or simultaneously with a positionally specific microbial lipase and a positionally non-specific lipase. Advantageously, a high degree of hydrolysis of the triglycerides can be obtained.

DETAILED DESCRIPTION OF THE INVENTION

Triglyceride and reaction products

The process of the invention can be applied to any oil or fat consisting largely of triglycerides (triacylglycerol), e.g. vegetable oils and animal fat, typically containing more than 90 % (e.g. more than 95 %) by weight of triglycerides. The acyl groups in the triglyceride may be linear fatty acyl groups, typically with 4-24 carbon atoms, particularly 12-22 carbon atoms. They may be saturated or unsaturated containing one or more double bonds. The triglyceride may particularly be a triglyceride of unsubstituted acyl groups, i.e. acyl groups of the general formula R-CO where R is a hydrocarbyl group.

The process can be conducted at moderate temperatures, and advantageously it can even be applied to thermolabile triglycerides, e.g. triglycerides with polyunsaturated acyl groups. The process leads to a high yield of glycerol and fatty acid which can be separated and purified by conventional methods. Advantageously, odor or off-flavor formation can largely be avoided because high temperatures are not used, making the fatty acid suitable for use in the food industry.

Lipases

The invention uses two lipases (triacylglycerol lipase), i.e. enzymes that catalyze the hydrolysis of ester bonds in triglycerides (triacylglycerol). They are classified as EC 3.1.1.3 according to Enzyme Nomenclature.

The two lipases are characterized by their positional specificity, i.e. the specificity for acyl groups in the 3 positions of a triglyceride. Thus, the microbial positionally specific (or 1,3-specific) lipase hydrolyzes acyl groups in the 1- and 3-positions with little or no activity in the 2-position, whereas the positionally non-specific lipase hydrolyzes acyl groups in all three positions at comparable rates.

The positional specificity of a lipase may be determined as described in WO 8802775, in WO 8901032 or in Example 8 of WO 9414940. The present invention uses a positionally non-specific lipase and a specific microbial lipase. Each lipase may be used in native (soluble) form or in immobilized form.

Positionally non-specific lipase

The non-specific lipase may be microbial, e.g. fungal or bacterial, particularly one derived from the following genera and species as described in the indicated publications: *Candida, C. rugosa* (also called *C. cylindracea*), *C. antarctica* lipase A or B (WO 8802775), *Pseudomonas, P. cepacia* (WO 8901032), *Streptomyces* (WO 9414940). It may also be a variant obtained by substitution, deletion or insertion of one or more amino acids in of one of the indicated lipases, e.g. as described in WO 9401541.

Positionally specific microbial lipase

The specific microbial lipase may be fungal or bacterial, e.g. derived from the following genera and species as described in the indicated publications: *Thermomyces, T. lanuginosus* (also known as *Humicola lanuginosa*, EP 305216, US 5869438), *Rhizomucor, R. miehei, Fusarium, F. oxysporum* (WO 9826057), or a lipase variant, e.g. as described in WO 9707202. The specific microbial lipase may also be a cutinase, i.e. an enzyme which also has cutinase activity (EC 3.1.1.74), e.g. a cutinase from *Humicola, H. insolens* (WO 9613580) or a cutinase variant, e.g. as described in WO 00/34450 or WO 0192502.

Immobilized lipase

The lipase may be immobilized, e.g., by adsorption on particulate silica, by covalent linkage with glutaraldehyde to particulate silica, by adsorption on a particulate macroporous weakly basic anion exchange resin, by adsorption on polypropylene or by cross-linking, particularly with glutaraldehyde, e.g. with addition of $MgSO_4$. The immobilization may be carried

out as described in EP 140452, WO 8902916, WO 9005778, WO 9015868, EP 232933 or US 4665028.

The two lipases may be mixed before immobilization, or they may be immobilized separately. In the latter case, the two immobilized lipases may be mixed, or they may be used separately in consecutive steps.

Hydrolysis process

The process may be carried out by contacting the triglyceride and water in a stirred tank with the two lipases in native (soluble) form or in immobilized form, or by continuously passing the triglyceride and water through one or more columns with a fixed bed of the lipases in immobilized form.

The stirred tank may be used batchwise or continuously. The effluent from the stirred tank (whether batchwise or continuous) may be separated into an oily phase containing triglyceride and fatty acid, and an aqueous phase containing glycerol and lipase. Water and lipase from the aqueous phase may be recycled, optionally after separation of glycerol, for better utilization of the lipase.

Typical conditions for stirred-tank operation are 30-60°C, particularly 40-55°C, and a reaction time from 6 to 72 hours, particularly 12 to 48 hours. The oil/water ratio from 1:2 to 3:1 (weight/weight) may be above 1:2, particularly above 1:1 , and may be below 5:1, particularly below 3:1 or below 2:1.

In a column process, the triglyceride and water may pass through a column containing the two lipases in immobilized form or through a series of two or more columns each containing a lipase in immobilized form. Thus, the triglyceride and water may pass through a first column with immobilized specific microbial lipase and a second column with immobilized non-specific lipase. The triglyceride and water may be subjected to high-shear mixing before entering the column or fed to the column from two separate substrate lines (co-current or countercurrent). Typical conditions for column operation are 30-80°C, particularly 40-70°C. The oil/water ratio from 1:2 to 3:1 (weight/weight) may be above 1:2, particularly above 1:1 , and may be below 5:1, particularly below 3:1 or below 2:1. Enzyme bed Height/Diameter ratios of column can be from 2-10, particularly 5-8. Residence times, which insures that linear velocity flow is greater than the critical linear velocity flow, can be used.

Advantageously, the process does not require the addition of a bile salt or other surfactant.

EXAMPLES

Example 1

Oil and water were treated in a tank with stirring with a combination of two lipases according to the invention. For reference, each lipase was tested alone at double dosage.

The positionally non-specific lipase was *Candida antarctica* lipase A, and the positionally specific microbial lipase was the lipase from *Thermomyces lanuginosus* or a cutinase variant disclosed in WO 00/34450. The enzyme combination included each enzyme at a dosage of 0.1 mg enzyme protein per g oil, and reference experiments were made with single enzymes at a dosage of 0.2 mg/g.

The conditions were 55°C, 450 rpm stirring, reaction time 24 hours, oil/water ratio 1:1 w/w. The degree of hydrolysis (DH) was determined from the acid value (AV) and saponification value (SV). % DH = (AV/SV) * 100 %. The results were:

	Specific lipase	Non-specific lipase	% DH
Invention	*T. lanuginosus* lipase, 0.1 mg/g	*C. antarctica* lipase A, 0.1 mg/g	94.2 %
	Cutinase variant, 0.1 mg/g	*C. antarctica* lipase A, 0.1 mg/g	98.0 %
Reference	*T. lanuginosus* lipase, 0.2 mg/g	-	77.0 %
	Cutinase variant, 0.2 mg/g	-	84.4 %
	-	*C. antarctica* lipase A, 0.2 mg/g	72.8 %

The results clearly show synergy, i.e. a higher degree of hydrolysis with the lipase combination compared to each lipase alone at double dosage.

Example 2

Oil was hydrolyzed with a lipase combination as in Example 1, except that the non-specific lipase was *Candida rugosa* lipase, different lipase dosages were used given in % by weight), and the temperature was 40°C. The results were:

	Microbial specific lipase	Non-specific lipase	% DH
Invention	*T. lanuginosus* lipase, 0.14 %	*C. rugosa* lipase, 0.05 %	93.3 %
Reference	*T. lanuginosus* lipase, 0.28 %	-	51.0 %
	-	*C. rugosa* lipase, 0.1 %	90.0 %

These results also show clear synergy, i.e. a higher degree of hydrolysis with the lipase combination compared to each lipase alone at double dosage.

Example 3

20 g of soy bean oil and 20 g of water were filled into a shake flask together with a positionally specific microbial lipase (*Thermomyces lanuginosus* lipase) and a non-specific lipase (*C. antarctica* lipase A) at dosages of 0.1 mg enzyme protein per g oil for each lipase. Reference tests were made separately with each lipase at double dosage. The flasks were shaken at 200 rpm and 50°C, and the degree of triglyceride hydrolysis was analyzed after 24 hours by titration with NaOH. The results were:

	Specific lipase	Non-specific lipase	% hydrolysis
Invention	0.1 mg/g	0.1 mg/g	95.7 %
Reference	0	0.2 mg/g	64 %
	0.2 mg/g	0	78 %

These results also show clear synergy, i.e. a higher degree of hydrolysis with the lipase combination compared to each lipase alone at double dosage.

CLAIMS

1. A process for hydrolyzing a triglyceride, comprising contacting the triglyceride and water consecutively or simultaneously with a positionally specific microbial lipase and a positionally non-specific lipase.

2. The process of claim 1 wherein the triglyceride and water are contacted simultaneously with the two lipases under stirring.

3. The process of claim 1 or 2 comprising passing the triglyceride and water continuously through a column holding the two lipases in immobilized form.

4. The process of any of claims 1-3 comprising passing the triglyceride and water consecutively through a first column holding the specific microbial lipase in immobilized form and a second column holding the non-specific lipase in immobilized form.